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1: Mol Cell Endocrinol 1984 Jul;36(3):165-73

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Transmembrane delivery of polypeptide hormones bypassing the intrinsic cell surface receptors: a conjugate of insulin with alpha 2-macroglobulin (alpha 2M) recognizing both insulin and alpha 2M receptors and its biological activity in relation to endocytic pathways.

Ito F, Ito S, Shimizu N.

125I-labeled insulin has been cross-linked to alpha 2-macroglobulin (alpha 2M) via a disulfide bond. The resulting insulin-alpha 2M conjugate carried 2.2 insulin moieties per mole of alpha 2M and was able to deliver insulin into rat hepatoma cells H35 and HTC. The insulin delivery was mediated predominantly through alpha 2M receptors and 2 h after binding it was found in the lysosomal fractions in the form of conjugate. When the conjugate was applied to rat hepatoma cells it stimulated activity of tyrosine aminotransferase (TAT) with a potency one-half that of native insulin. Hepatoma cells which were treated with conjugate in the presence of bacitracin were also stimulated for TAT activity. Since bacitracin completely inhibited the alpha 2M binding to its receptors, but inhibited conjugate binding by only 80%, this stimulation must have resulted from the remaining binding of conjugate. These results indicate that the insulin-alpha 2M conjugate was biologically active if it bound to insulin receptors, but that the conjugate bound and internalized through alpha 2M receptors did not act as a mediator for TAT activation. Our results using Percoll density gradients indicate a difference in intracellular processing between insulin, alpha 2M and the conjugate. Mechanisms of action of the conjugate are discussed in relation to the receptor-mediated endocytic pathways.

PMID: 6205913 [PubMed - indexed for MEDLINE]

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1: Biochem Biophys Res Commun 1995 Nov 2;216(1):399-405

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Receptor-linked antigen delivery system. Importance of autologous alpha 2-macroglobulin in the development of peptide vaccine.

Mitsuda S, Nakagawa T, Nakazato H, Ikai A.

Department of Biological Sciences, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama, Japan.

We have hijacked a process of the receptor-mediated endocytosis to transport peptide antigens into antigen presenting cells (APCs) for the purpose of increasing the level of antigen presentation (named Receptor-Linked Antigen Delivery System (R-LADS)). By coupling an endogenous plasma proteinase inhibitor alpha 2-macroglobulin (alpha 2M) to a synthetic peptide having a partial sequence of HIV-1 envelope protein, alpha 2M was made to carry the peptide into APCs as a part of the normal alpha 2M cycle, which resulted in an increased production of specific antibodies against the peptide (Mitsuda, S., Nakagawa, T., Osada, T., Shimamoto, T., Nakazato, H. and Ikai, A. (1993) Biochem. Biophys. Res. Commun. 194, 1155-1160). We demonstrate here that this procedure becomes a more efficient tool for antibody production when autologous transporter protein was used. By using murine alpha 2M (m alpha 2M) instead of heterologous human alpha 2M (h alpha 2M) when mice were experimental animals, we were able to dramatically enhance the production level of anti-HIV-1 peptide antibodies and shorten the period which is needed for antibody production. We aim to develop effective peptide vaccines by further improving this system.

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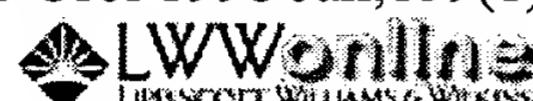
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1: J Urol 1998 Jan;159(1):297-303

Related Articles, Links



Prostate-specific antigen forms complexes with human alpha 2-macroglobulin and binds to the alpha 2-macroglobulin receptor/LDL receptor-related protein.

Otto A, Bar J, Birkenmeier G.

Institute of Biochemistry, University of Leipzig, Germany.

PURPOSE: To investigate the binding of the prostate-specific antigen (PSA) to human alpha 2-macroglobulin (alpha 2-M) and to alpha 1-antichymotrypsin (ACT). **MATERIALS AND METHODS:** Binding analysis was evaluated by electrophoresis, Western-blotting, enzyme-linked immunosorption assay (ELISA) and size exclusion chromatography. Quantification of PSA and of different forms of alpha 2-M was performed using commercial test kits. The cleavage site of PSA in alpha 2-M was analyzed by SDS-PAGE and microsequencing. **RESULTS:** Binding of PSA to alpha 2-M is initiated by the cleavage of the peptide bond between amino acids Tyr 686 and Glu 687 of the bait region indicating a chymotrypsin-like activity of the PSA. The PSA's proteolytic cleavage triggers the transformation of alpha 2-M as detected by conformation-specific monoclonal antibodies. Kinetic analysis revealed faster binding of PSA to alpha 2-M than to ACT. The PSA bound to alpha 2-M is caged by the inhibitor and thus escapes detection by antibodies. This results in an incorrect calculation of the level of PSA when released from prostate into the blood. Complexes of PSA-alpha 2-M and PSA-ACT were found to bind to the alpha 2-macroglobulin receptor/LDL receptor-related protein (alpha 2-M-R/LRP) which may be the clearance receptor for PSA.

CONCLUSIONS: Quantifying free PSA and PSA-ACT complexes, as routinely done in managing prostate-associated diseases, does not represent the total secretion capacity of the prostate. The proteinase inhibitor alpha 2-M has to be considered as a main contributor to PSA complex formation in the blood.

PMID: 9400498 [PubMed - indexed for MEDLINE]

References (20)

PROSTATE-SPECIFIC ANTIGEN FORMS COMPLEXES WITH HUMAN alpha 2-MACROGLOBULIN AND BINDS TO THE alpha 2-MACROGLOBULIN RECEPTOR/LDL RECEPTOR-RELATED PROTEIN

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From the Institute of Biochemistry, University of Leipzig, Leipzig, Germany.

Accepted for publication August 20, 1997.

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THE JOURNAL OF UROLOGY 1998;159:297-303

Abstract

Purpose: To investigate the binding of the prostate-specific antigen (PSA) to human alpha 2-macroglobulin (alpha 2-M) and to alpha 1-antichymotrypsin (ACT).

Materials and Methods: Binding analysis was evaluated by electrophoresis, Western-blotting, enzyme-linked immunosorption assay (ELISA) and size exclusion chromatography. Quantification of PSA and of different forms of alpha 2-M was performed using commercial test kits. The cleavage site of PSA in alpha 2-M was analyzed by SDS-PAGE and microsequencing.

Results: Binding of PSA to alpha 2-M is initiated by the cleavage of the peptide bond between amino acids Tyr 686 and Glu 687 of the bait region indicating a chymotrypsin-like activity of the PSA. The PSA's proteolytic cleavage triggers the transformation of alpha 2-M as detected by conformation-specific monoclonal antibodies. Kinetic analysis revealed faster binding of PSA to alpha 2-M than to ACT. The PSA bound to alpha 2-M is caged by the inhibitor and thus escapes detection by antibodies. This results in an incorrect calculation of the level of PSA when released from prostate into the blood. Complexes of PSA-alpha 2-M and PSA-ACT were found to bind to the alpha 2-macroglobulin receptor/LDL receptor-related protein (alpha 2-M-R/LRP) which may be the clearance receptor for PSA.

Conclusions: Quantifying free PSA and PSA-ACT complexes, as routinely done in managing prostate-associated diseases, does not represent the total secretion capacity of the prostate. The proteinase inhibitor alpha 2-M has to be considered as a main contributor to PSA complex formation in the blood.

Key Words: prostate-specific antigen, alpha 2-macroglobulin, alpha 1-antichymotrypsin, alpha 2-macroglobulin receptor/LDL receptor-related protein

The main clinical interest in PSA has to do with its remarkable value as a serum marker for carcinoma of

the prostate [1,2] and for breast cancer. [3] However, the diagnostic potential of PSA in malignancy is limited by the increase of PSA concentration in benign prostatic hyperplasia. [4] One way to enhance diagnostic specificity is to combine the detection of complexed PSA in addition to free PSA. [5] In serum, PSA occurs predominantly in complex with alpha 1-antichymotrypsin (ACT). [6] PSA was also found to form complexes with two other abundantly occurring proteinase inhibitors of the plasma, alpha 2-macroglobulin (alpha 2-M) and alpha 1-proteinase inhibitor. [7] Currently, these complexes were only detectable in small amounts in cancerous sera with elevated PSA levels and are assumed to be negligible. alpha 2-M has not been seriously considered as a main contributor for PSA complex formation.

alpha 2-M is a pan-proteinase inhibitor with the capability of inhibiting a large variety of proteinases. After proteolytic cleavage of the "bait" region, the proteinase is entrapped and loses most of its activity, at least toward high molecular weight substrates. [8] The concomitant interruption of the thiolester triggers further biological functions of the inhibitor such as binding cytokines, growth factors and hormones, [9,10] as well as clearance by the alpha 2-M-R/LRP present on the surface of different cells. [11,12,13] It is suggested that alpha 2-M-R/LRP plays an important role in the regulation of proteolytic activity in tissue and pericellular space. [14]

In this study, we have analyzed the interaction of PSA with alpha 2-M and ACT. Binding of PSA to alpha 2-M contributes significantly to that portion of PSA found in complexes in serum. We will further demonstrate that PSA, either in complex with alpha 2-M or with ACT, is bound to cellular alpha 2-M-R/LRP.

MATERIALS AND METHODS

Materials.

HRP-labeled rabbit anti-alpha 2-M-Ig was obtained from BioMac (Leipzig, Germany). Human ACT, HRP-labeled goat anti-rabbit Ig and DAB were gifts from Serva (Heidelberg, Germany). Rabbit anti-human ACT-Ig was obtained from Dako (Hamburg, Germany). Microtiter 96-well plates were from Nunc (Wiesbaden, Germany). Streptavidine-labeled HRP was from Sigma (Heidelberg, Germany). Plasma was obtained from citrated blood from a 25-year-old female healthy volunteer. Purified alpha 2-M-R/LRP isolated from human placenta, human native alpha 2-M, alpha 2-M-MA, and alpha 2-M-CT were obtained from BioMac.

Methods: protein purification.

PSA was purified from seminal fluid according to Lilja. [1] The final product was homogenous as judged by electrophoresis and immunoblotting. The sequence analysis yielded NH₂-Ile-Val-Gly-Gly-Trp-Glu-Leu-Glu-Lys-His-, which coincides, with exception of position 7 (Leu-Cys), with the N-terminal sequence of mature PSA.

Electrophoresis.

Resolution of native and transformed alpha 2-M was achieved by rate electrophoresis under nondenaturating conditions on 5% polyacrylamide slab gels. [15,16] Sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) was performed in polyacrylamide slab gel gradients using the buffer system according to Laemmli's method. [17] Protein staining was accomplished with Coomassie Brilliant Blue R 250.

Quantitation of alpha 2-M and PSA.

Quantitation of total and transformed alpha 2-M was performed by ELISA using two different monoclonal antibodies directed against alpha 2-M. The ELISA test kits "Macro Nat" and "MacroTrans" were purchased from BioMac. The ELISA test kit "Enzymun-Test PSA" from Boehringer (Mannheim, Germany) was used for quantitation of PSA.

Radiolabeling.

Labeling of 10 micro g. PSA was accomplished with [¹²⁵I]Na (Amersham, Braunschweig, Germany) using the chloramine-T method according to Hunter and Greenwood's method. [18] The specific activity of [¹²⁵I]PSA ranged from 70 to 80 Ci./gm. For preparing radiolabeled alpha 2-M-PSA and ACT-PSA complexes, freshly labeled [¹²⁵I]-PSA was reacted with native alpha 2-M (molar ratio 5:1) and with ACT (molar ratio 2:1) for 1 hour at 37C in PBS (50 mM. sodium phosphate, 150 mM. NaCl, pH 7.4) and then gel filtrated to remove free PSA.

Binding studies using SEC.

Mixtures of unlabeled and [¹²⁵I]-labeled PSA were incubated with native alpha 2-M or ACT in PBS at 37C for 1 hour and chromatographed on a Bio-Sil SEC 125 (7,8 x 300 mm.) column (Bio-Rad) equilibrated with PBS at a flow rate of 1 ml. per minute. Radioactivity was detected by gamma-counting aliquots of the fractions, and the distribution of PSA was determined by ELISA.

Immunoblotting PSA.

For immunoblotting, proteins were separated by SDS-PAGE under nonreducing conditions, electroblotted to nitrocellulose acetate membranes (Schleicher & Schuell, Dassel, Germany) and blocked for 2 hours at 20C with 5% defatted milk powder in PBS. The membranes were then incubated with the mouse monoclonal biotinylated anti-PSA antibody (Enzymun-Test-PSA, Boehringer), diluted 1:10 with PBS-T (50 mM. sodium phosphate, 150 mM. NaCl, 0.05% Tween 20, pH 7.4) containing 1% BSA and, finally, with streptavidine-labeled HRP, diluted 1:1000 in the same buffer. The bands were visualized by DAB and H₂O₂ as substrates.

Binding studies on immobilized receptor.

Purified receptor (2 micro g./ml. 0.1 M. NaHCO₃, pH 9.15) was coated on microtiter plates overnight and then incubated with increasing concentrations of proteinase-inhibitor complexes in PBS-T for 3 hours at 37C. After washing, the bound complexes were determined by means of specific polyclonal antibodies. The binding curves were analyzed according to the equation:

$$A = A_{\max} (L)/[(K_d + (L)]$$

where A is the absorbance at 492 nm, A_{max} is the maximum absorbance at saturation conditions, L is the molar concentration of the free ligand or proteinase-complexed ligand, respectively, and K_d is the dissociation constant, making the assumption that the amount of added ligand is greater than the amount of receptor bound to the microtiter wells.

Amino acid sequence analysis.

The N-terminal sequence was determined according to Edman's procedure with an Applied Biosystems 473

A sequencer. Protein bands of SDS-PAGE were electroblotted onto a PVDF membrane (Pro Blot membrane) according to the manufacturer's instructions. After staining with Coomassie Brilliant Blue R 250 in methanol/water/acetic acid (40/49/1), destaining in methanol/water (50/50) and drying, the band of interest was cut off and subjected to the reaction cartridge of the sequencer.

RESULTS

Conformational transition of alpha 2-M induced by PSA.

Complex formation between native alpha 2-M and PSA causes a conformational change in the inhibitor which can be recognized by rate electrophoresis (Figure 1). The PSA-transformed alpha 2-M is located between the slow (native alpha 2-M) and the fast (alpha 2-M-CT) form of the inhibitor indicating that PSA is unable to convert alpha 2-M completely from the slow into the fast-moving form under given conditions. The degree of transformation of alpha 2-M was quantified by ELISA using conformation specific monoclonal antibodies (Figure 2). The monoclonal antibody alpha-1 used is known to recognize an epitope on a C-terminal peptide stretch hidden in native, but exposed in the transformed inhibitor. [16] As seen, in comparison to alpha 2-M-CT, only approximately 50% of native inhibitor was transformed by PSA. These data coincide with the intermediate mobility of the alpha 2-M-PSA-complexes in rate electrophoresis as shown in Figure 1.

Figure 1. Rate electrophoresis of alpha 2-M in presence of PSA. Rate electrophoresis was performed in 5% polyacrylamide gels. Proteins were run at 200 V for 2 hours in a Miniprotean apparatus from BioRad and stained with 0.05% Coomassie Brilliant Blue G 250 in trichloroacetic acid (12.5%). All incubations were made in PBS in a volume of 20 micro L. Lane 1: 3 micro g. of native alpha 2-M (slow-moving form) without PSA. Lane 2: 3 micro g. of native alpha 2-M incubated at 37C for 1 hour with PSA at a molar ratio of 5:1 (PSA/alpha 2-M.). Lane 3: 3 micro g. of native alpha 2-M incubated with alpha-chymotrypsin for 2 minutes at a ratio of 2:1 (CT/alpha 2-M)(fast-moving form).

Figure 2. Evaluation of the transformation of alpha 2-M by PSA. Microtiter plates were coated with transformation-specific monoclonal antibody alpha-1(2 micro g./ml.). After intensive washing with PBS-T, plates were incubated 1 hour at 37C with 45 nM. of alpha 2-M-CT; 45 nM. of alpha 2-M-PSA (molar ratio of 1:10), 45 nM. of alpha 2-M-PSA (molar ratio of 1:5); 45 nM. of alpha 2-M-PSA (molar ratio of 1:1), and 45 nM. of native alpha 2-M, respectively. Incubation volume was 200 micro L. Reaction of alpha 2-M with different concentrations of PSA was accomplished in PBS for 1 hour at 37C. After washing, plates were incubated with HRP-labeled rabbit anti-human alpha 2-M polyclonal antibody (1:1000 diluted) in PBS-T for 1 hour at 37C. Plates were washed and developed with POD and H₂O₂. Maximum binding achieved with totally transformed alpha 2-M-CT was set at 100%.

Cleavage pattern of alpha 2-M-PSA interaction.

As for other proteinases, the transition of alpha 2-M is initiated by cleavage of a peptide bond in the bait region. This is demonstrated in Figure 3A. PSA was incubated with native alpha 2-M and the bait region cleavage pattern was evaluated after subjecting the samples to SDS-PAGE under reducing conditions. In

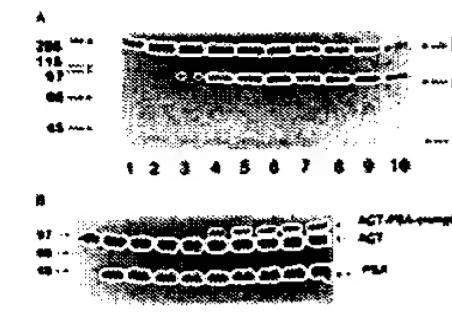
the presence of a 5 molar excess of PSA, a polypeptide fragment with an apparent molecular mass of 93 to 95 kDa (band III) became apparent. Increasing the molar ratio did not result in the 180 kDa alpha 2-M monomer (band II) cleaving further. During the course of the reaction, a faint double band with an apparent molecular mass of approximately 400 kDa (band I) appeared, strongly suggesting that this may represent cross-linked reaction products between PSA and alpha 2-M. In contrast to the typical alpha-chymotryptic digest, an incomplete cleavage of the 180 kDa polypeptide of alpha 2-M by PSA was observed. This indicates that probably only one dimer of the inhibitor is attacked by PSA. Higher resolution of the 93 to 95 kDa cleavage products was achieved by SDS-PAGE in 6% gel (Figure 3B). At this point, the sample in lane 3 could be resolved into two polypeptides (band IIIa and IIIb) with an apparent molecular mass of 93 kDa and 95 kDa. After blotting onto PVDF-membranes, these polypeptides were eluted and sequenced in an amino acid sequencer. The N-terminal sequence of the 93 kDa polypeptide was determined as NH₂-Ser-Val-Ser-Gly-Lys-Pro-Gln-Tyr-Met-Val-, which represents the N-terminus of the 180 kDa monomer of alpha 2-M. The N-terminal sequence of the 95 kDa polypeptide was NH₂-Glu-Ser-Asp-Val-Met-Gly-Arg-Gly-His, indicating that PSA cleaves the peptide bond within the bait region between Tyr 686 and Glu 687. When PSA was reacted with alpha 2-M-MA under the same conditions, only small fragmentation of the 180 kDa subunit was observed, indicating that the bait region in the transformed inhibitor is not accessible (not shown).

Figure 3. SDS-PAGE analysis of alpha 2-M-PSA reactions. A, 10 micro g. of alpha 2-M were incubated with 5-molar excess of PSA in PBS at 37C for 1 hour and subjected to electrophoresis in SDS-PAGE gradients (3 to 15%) under reducing conditions. Gels were stained with Coomassie Brilliant Blue R 250. Molecular weight marker proteins used were: carbonic anhydrase (29 kDa); ovalbumin (45 kDa); albumin (66 kDa); phosphorylase b (97 kDa); beta-galactosidase (116 kDa), myosin (205 kDa). Lane 1: native alpha 2-M (5 micro g.); lane 2: alpha 2-M-CT (10 micro g.); lane 3: alpha 2-M-PSA (10 micro g.); lane 4: PSA (3 micro g.). Band I: alpha 2-M-PSA aggregates; band II: uncleaved 180 kDa alpha 2-M. monomer; band III: bait region cleavage products; band IV: PSA. B, 5 micro g. of alpha 2-M-PSA (sample 3) were subjected to SDS-PAGE (6% polyacrylamide) under reducing conditions for better resolution of band III.

Complex formation of PSA with alpha 2-M and ACT.

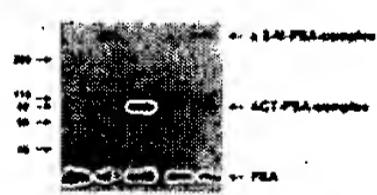
(Figure 4) demonstrates the time-dependence of the interaction of PSA with alpha 2-M in comparison to PSA-ACT complex formation. This was evaluated by measuring the bait region cleavage reaction (band III) in the case of alpha 2-M (Figure 4A) or by following up the formation of the 100 kDa PSA-ACT complex (Figure 4B). The occurrence of these bands in SDS-PAGE may be described by second order kinetics exhibiting a biphasic curve for PSA-alpha 2-M reaction with rate constants of $k_1 = 2,3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ and $k_2 = 1.9, \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$. The reaction of PSA with ACT ($k = 0,87 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$) was found to proceed more slowly compared with alpha 2-M.

Figure 4. Time course of interaction of PSA with alpha 2-M and ACT. PSA was incubated with alpha 2-M (A) and ACT (B) at a PSA/inhibitor ratio of 5:1 and 2:1, respectively, in PBS at 37C. At certain time intervals aliquots of 10 micro g. were removed, immediately denatured by boiling (5 minutes) in SDS-incubation buffer containing beta-mercaptoethanol and subjected to SDS-PAGE (3 to 20% gradient gels). Proteins were stained with Coomassie Brilliant Blue G-250. Relative portions of the 93 to kDa band (band III) of alpha 2-M and the 100 kDa band (ACT-PSA complex), respectively, were analyzed by scanning in a densitometer with automatic integrator. Incubation time: 1, no PSA; 2 1 minute; 3, 5 minutes; 4, 10 minutes; 5, 20 minutes; 6, 30 minutes; 7, 45 minutes; 8, 60 minutes; 9, 90 minutes; 10, 120 minutes. Labeling of bands and molecular weight marker protein used were the same as in Figure 3.



Western blotting revealed that PSA forms SDS-stable complexes with alpha 2-M and ACT which can be recognized by monoclonal antibodies directed against PSA (Figure 5). PSA immunoreactivity was associated with the 360 kDa polypeptide of alpha 2-M dimer and with aggregates of higher molecular mass (lane 2). As expected, PSA formed a complex with ACT with an apparent molecular mass of 100 kDa (lane 3). We raised the question of the ratio of distribution of PSA when both inhibitors were present. Thus, the concentrations of the inhibitors were adjusted to simulate conditions found in plasma (lane 4). Although no exact quantitative data can be deduced from the Western-blot experiments, there are no indications of a preferable binding of PSA to ACT. To examine whether these complexes are also formed in blood, fresh plasma was mixed with PSA (Lane 5). The result clearly indicate that PSA, when entering the blood, is bound to ACT as well as to alpha 2-M.

Figure 5. Western blot analysis of PSA-inhibitor complexes. PSA was incubated with alpha 2-M (molar ratio of 5:1), with ACT (molar ratio 2:1), with mixtures of alpha 2-M and ACT in PBS, and with fresh human plasma, respectively, for 1 hour at 37C. After SDS-PAGE in 3 to 15% gradient gels under non-reducing conditions, polypeptides were blotted onto nitrocellulose acetate membranes which were blocked afterward by defatted milk powder (5%). Membranes were then incubated with biotin-labeled monoclonal antibody directed against PSA overnight at 4C and finally with streptavidin-labeled HRP. Lane 1: PSA (3 micro g.); lane 2: alpha 2-M (10 micro g.) plus PSA (3 micro g.); lane 3: ACT (6 micro g.) plus PSA (3 micro g.); lane 4: alpha 2-M (10 micro g.) and ACT (2 micro g.) plus PSA (3 micro g.); lane 5: PSA (3 micro g.) plus fresh plasma (1 micro L.). Molecular weight markers used were the same as in Figure 3.



SEC was applied to studying complex formation under native conditions (Figure 6). After incubating ACT or alpha 2-M with PSA spiked with ¹²⁵I-labeled PSA, the complexes were separated and the distribution of PSA was determined by ELISA and gamma-counting. As seen in Figure 6A, free PSA and PSA in complex with ACT could be immunologically detected. In contrast, the fraction of PSA bound to alpha 2-M escaped detection by the monoclonal antibodies (Figure 6B). However, ¹²⁵I-labeled PSA was found in association with the alpha 2-M peak. This indicates that PSA is presumably caged by the inhibitor and is not available for immunological detection under native conditions. Making a rough estimate of the distribution of radioactivity in SEC experiments gave rise to the assumption of a 1:1 complex formed under experimental conditions. PSA was found to also form complexes with the transformed inhibitor, but to a lower extent (Figure 6C). Continuing these experiments, we studied the effect of alpha 2-M and ACT on quantitation of

PSA in solution (see Table 1). It becomes apparent that, in the presence of native alpha 2-M, a significant fraction of PSA could not be recovered irrespective of whether purified alpha 2-M or fresh plasma was used. In contrast, forming complexes with ACT had no effect on immunological quantitation of PSA. The experiments further demonstrate that alpha 2-M which was already transformed by methylamine did not interfere with the immunological assay for PSA.

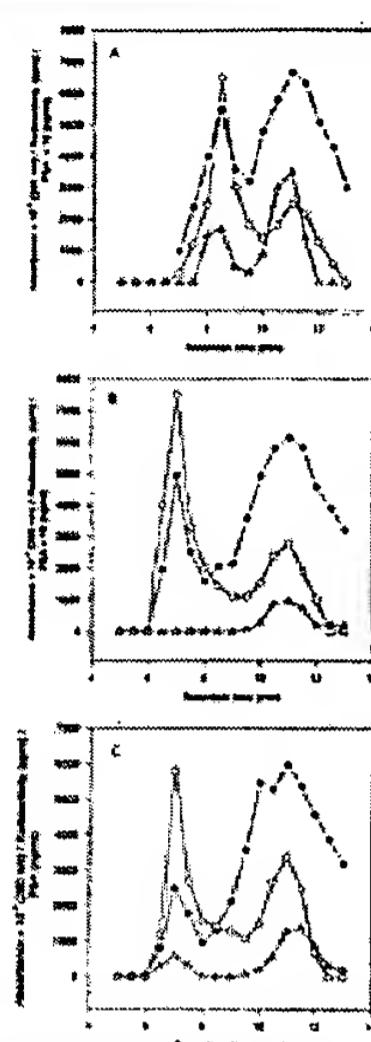


Figure 6. Size exclusion chromatography of PSA-inhibitor complexes. 50 micro g. of PSA spiked with ¹²⁵I-labeled PSA (100.000 cpm) was incubated with ACT (100 micro g.) (A) or alpha 2-M (218 micro g.) (B) and with alpha 2-M-MA (218 micro g.) (C) for 1 hour at 37C in PBS. Samples were run on Bio-Sil SEC 125 (7.8 x 300 mm.) (BioRad) column equilibrated with incubation buffer. Fractions of 0.5 ml. were collected and aliquots were analyzed for radioactivity ([shaded circle]), PSA (ELISA) ([triangle]), and protein at 280 nm ([circle]). Elution times for free alpha 2-M, free ACT and free PSA were 7 minutes, 8.5 minutes and 11.1 minutes, respectively.

Table 1. PSA concentration in samples

Binding of PSA-inhibitor complexes to immobilized alpha 2-M-R/LRP.

The results in Figure 2 demonstrate that PSA is capable of triggering the exposure of receptor-recognition sites in the alpha 2-M molecule. This causes a strong binding of the alpha 2-M-PSA complex to the immobilized receptor, which is almost comparable to the binding of alpha 2-M-MA (Figure 7A). The binding to the receptor was found to be specific because it could be competitively inhibited by an excess of unlabeled alpha 2-M-PSA (Figure 7B). The inhibiting effect of EDTA emphasizes the fact that Ca sup ++-ions are required for receptor binding. Analogous experiments were performed with ACT-PSA complexes (Figure 8). Surprisingly, the ACT-PSA complex was found to be recognized by the immobilized alpha 2-M-R/LRP as well. Rough evaluation of binding constants from the binding curves (Figure 7A and Figure 8A) revealed that the dissociation constant for receptor binding of alpha 2-M-PSA ($K_d = 0.91 \pm 0.15$ nM.) is approximately one order of magnitude lower compared with binding of ACT-PSA ($K_d = 12.04 \pm 1$ nM.). Control experiments were performed showing that the binding of ACT-PSA complex was not mediated by PSA, demonstrating that the receptor recognition site of the complex is located on the ACT molecule. Binding studies with radio-labeled ACT-PSA complexes revealed that binding to the receptor could be relieved by an excess of unlabeled complex, but not by alpha 2-M-MA (Figure 8B). This suggests the existence of a separate binding site in the receptor for ACT-proteinase complexes.

Figure 7. Binding of alpha 2-M-PSA to immobilized receptor. A, microtiter plates were coated with alpha 2-M-RLRP (2 micro g./ml.) overnight at 4C. After washing increasing concentrations of alpha 2-M, alpha 2-M-MA and alpha 2-M-PSA complexes which were dissolved in 200 micro L. of 20 mM. Hepes, 5 mM. Ca sup ++, 150 mM. NaCl, 0.05% Tween-20, pH 7.5 were incubated with immobilized receptor for 3 hours at 37C. After washing, bound alpha 2-M was detected by incubation with HRP-labeled rabbit anti-alpha 2-M-Ig (1: 1000 diluted in PBS-T). ([circle]) native alpha 2-M; ([square]) alpha 2-M-PSA (reacting molar concentrations were 1:5); alpha 2-M-MA ([shaded circle]). B, microtiter plates were coated with alpha 2-M-R/LRP (2 micro g./ml.). After washing, plates were incubated with ¹²⁵ I-labeled alpha 2-M-PSA (5 nM.) dissolved in 20 mM. Hepes, 5 mM. Ca sup ++, 5 mM. Mg sup ++, 150 mM. NaCl, 0.05% Tween-20, 3% BSA, pH 7.4 or in 20 mM. Hepes, 20 mM. EDTA, pH 7.4., respectively, for 3 hours at 37C. After washing, plates were incubated with 0.7% SDS, 1 M. NaOH for 12 hours to remove bound radioactivity. Finally, samples were analyzed by gamma-counting. Column 1:¹²⁵ I-labeled alpha 2-MPSA (5 nM.); Column 2:¹²⁵ I-labeled alpha 2-M-PSA (5 nM.) plus unlabeled alpha 2-M-PSA (100 molar excess); Column 3:¹²⁵ I-labeled alpha 2-M-PSA (5 nM.) plus 20 mM. EDTA; Column 4:¹²⁵ I-labeled alpha 2-M-PSA (5 nM.) incubated with uncoated plates.

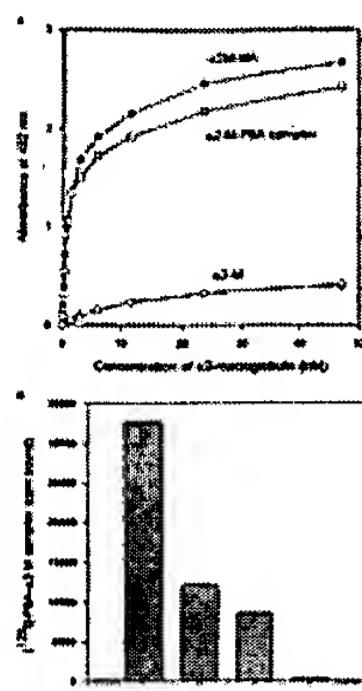
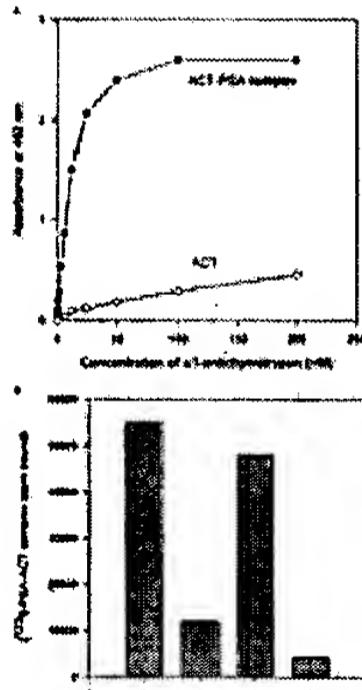


Figure 8. Binding of ACT-PSA to immobilized receptor. A, microtiter plates were coated with alpha 2-M-R/LRP (2 micro g./ml.). After washing, increasing concentrations of ACT and ACT-PSA complex dissolved in PBS-T were incubated with immobilized receptor for 3 hours at 37C. After washing, plates were incubated with rabbit anti-ACT-Ig (1:1000 diluted) for 1 hour at 37C. After washing, detection was accomplished by incubation with HRP-labeled goat anti-rabbit Ig (1:1000 diluted) for 1 hour at 37C. ([circle]) ACT; ([shaded circle]) ACT-PSA (reacting molar concentrations were 1:2). B, microtiter plates were coated with alpha 2-M-R/LRP (2 micro g./ml.). After washing, plates were incubated with ¹²⁵ I-labeled ACT-PSA (10 nM.) dissolved in 20 mM. Hepes, 5 mM. Ca sup ++, 5 mM. Mg sup ++, 150 mM. NaCl, 0.05% Tween-20, 3% BSA, pH 7.4 for 3 hours at 37C. After washing plates were incubated with 0.7% SDS, 1 M. NaOH for 12 hours to remove bound radioactivity. Finally, samples were analyzed by gamma-counting. Column 1:¹²⁵ I-labeled ACT-PSA (10 nM.); Column 2:¹²⁵ I-labeled ACT-PSA (10 nM.) plus unlabeled ACT-PSA (100 molar excess); Column 3: ¹²⁵ I-labeled ACT-PSA (10 nM.) plus 200 nM. alpha 2-M-MA; Column 4:¹²⁵ I-labeled ACT-PSA (10 nM.) incubated with uncoated plates.



DISCUSSION

PSA proteolytically attacks alpha 2-M by a cleaving of the peptide bond between Tyr 686 and Glu 687. This site, which is identical to the cleavage site of alpha-chymotrypsin, is located within the bait region (amino acids 603 to 702) known to harbor the proteolytic sites of most proteinases. [19] The specificity of cleavage clearly indicates that PSA has chymotrypsin-like activity. [7] Similar to the action of other proteinases, the proteolytic cleavage of alpha 2-M by PSA results in a major conformational change. The incapability of PSA to totally transform the inhibitor, as demonstrated in Figure 1. and Figure 2, may be best explained by a slow reaction rate or by incomplete cleavage of alpha 2-M subunits as also found in

case of thrombin ($k = 3.2 \times 10^3$ M⁻¹ s⁻¹). [20] The biphasic kinetics found for conversion of alpha 2-M by PSA suggests that in an initial reaction a limited number of subunits, probably that of one dimer, is cleaved, followed by a nonproductive cleavage of the remaining subunits in a slower reaction. A similar mechanism has been previously discussed for thrombin-alpha 2-M interaction. [20] Indeed, alpha 2-M transformation by PSA could be completed by increasing reaction time (for example for 20 hours) rather than by increasing the concentration of the proteinase. The excess of required proteinase (PSA) for obtaining optimal transformation of alpha-2-M may indicate that the isolated PSA may be only 30 to 50% active.

PSA was found to form SDS-stable complexes with alpha 2-M. However, only about 1 to 5% of PSA became covalently attached, as deduced by autoradiography (not shown). The main portion of PSA seems to be non-covalently associated with alpha 2-M in a cage-like structure. This binding mode requires an active proteinase and the native inhibitor. The binding of PSA to alpha 2-M-MA (Figure 6) without concomitant bait region cleavage worth mentioning gives rise to the assumption of a second binding mode which may be associated with the expression of new binding sites on the surface of the inhibitor when it is transformed. A binding site such as this in transformed alpha 2-M has been recently described for human growth hormone [10] and would explain the inconsistencies in the literature concerning the immunological detection of PSA in alpha 2-M-PSA complexes. [21,22] Thus, PSA bound to transformed alpha 2-M is believed to be detectable by antibodies in contrast to PSA with bait region activity. This could also explain binding of nicked PSA to the inhibitor as recently described. [21] The data indicate that one has to be aware of the native structure of alpha 2-M used for in vitro experiments. In vivo, PSA is suggested to complex with alpha 2-M mainly via the bait region cleavage mode due to the high abundance of the native inhibitor in blood. [16]

We clearly have been able to demonstrate that in vitro PSA forms complexes with both ACT and alpha 2-M. Binding to ACT was found to be slower than to alpha 2-M. This suggests that in plasma where the levels of ACT (0.5 mg./ml.) and alpha 2-M (2.54 mg./ml.) [25] are 10^5 to 10^2 times greater than PSA (<2 ng./ml.) a significant part (approximately 50% to 60%) of the enzymatically active proteinase is expected to be complexed to alpha 2-M. Similar results have been demonstrated recently. [21] This shows that if both free PSA and PSA-ACT are simultaneously determined, it increases the predictability of benign and malignant tumors in men; [6] however, it does not reflect the secretion capacity of tumor cells per se. The fraction of PSA complexed to alpha 2-M has to be seriously considered in future prostate diagnosis management. However, the incapability of many currently commercially available immunoassays [4] to detect alpha 2-M-PSA complexes has obviously led to an underestimation of the diagnostic importance of this parameter. Currently, the origin of PSA-inhibitor complexes is not yet clear. ACT was found to be synthesized in PSA-producing cells as well. [12] There is no evidence of whether ACT in ACT-PSA complexes is predominantly derived from prostate epithelium or from the liver. This question may be addressed also for alpha 2-M-PSA complexes.

As a new finding, we most probably identified the receptor for PSA-inhibitor complexes, alpha 2-M-R/LRP in the human. The most striking result from the in vitro binding studies was that ACT needs to be activated by PSA to be effectively bound to the receptor. It is likely that this activation triggers the expression of receptor recognition sites similarly as for alpha 2-M. Although binding and clearance studies with cells should be done, these preliminary data suggest that PSA is most probably cleared via the alpha 2-M-R/LRP. At present, a clearance receptor for ACT-PSA complexes has not been described. Recently, it has been reported that in the human, the liver is the main site of elimination of PSA from circulation. [23] Hepatocytes are known to express to a great extent alpha 2-M-R/LRP, contributing mainly to the clearance of alpha 2-M-proteinase complexes from circulation. [14]

One function of PSA is discussed in connection with liquification of seminal fluid. [1] This effect of PSA

in seminal fluid may differ from PSA's proteolytic activity in the prostate or metastatic sites. Recently, the insulin-like growth factor binding protein 3 was found to be cleaved by PSA as well. This leads to an increased of biologically active IGF-1, which is known as a growth stimulator for prostatic epithelial cells. [24] Proteinase inhibitors such as ACT and alpha 2-M, which may regulate the activity of PSA in pericellular space, may thus indirectly affect growth and differentiation of PSA-producing cells or surrounding cells. In this context, we recently found a strong correlation between sperm cell quality and the concentration of alpha 2-M and the binding protein for IGF-1 [25] In addition, receptors mediating the clearance of PSA-proteinase inhibitor complexes, such as alpha 2-M-R/LRP, are suggested to play an important role in proteolysis-mediated cellular events. [14] We may expect from the detection of alpha 2-M-R/LRP on prostate carcinoma cells [26] that this receptor is involved in the regulation of growth and metastasis of prostate tumor cells.

In conclusion, PSA from epithelial prostate cells forms complexes with ACT as well as with alpha 2-M when entering the blood. The fact that PSA in complex with alpha 2-M has escaped immunological detection may have a great impact on the evaluation of current results regarding the quantitation of PSA under diverse clinical conditions. Tests should be developed which will take the fraction of PSA which is complexed to the alpha 2-M in blood into consideration.

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REFERENCES

1. Lilja, H.: A kallikrein-like serine protease in prostatic fluid cleaves the predominant seminal vesicle protein. *J. Clin. Invest.*, 76: 1899, 1985.
2. Stamey, T. A., Yang, N., Hay, A. R., McNeal, J. E., Freiha, F. S., and Redwine, E.: Prostate-specific antigen as a serum marker for adenocarcinoma of the prostate. *N. Engl. J. Med.*, 317: 909, 1987.
3. Diamandis, E. P., Yue, E. and Sutherland, D. J.: Detection of prostate-specific antigen immunoreactivity in breast tumors. *Breast Cancer Res. Treat.*, 32: 301, 1994.
4. Wu, T. J.: Assay for prostate-specific antigen (PSA). Problems and possible solutions. *J. Clin. Lab. Analysis*, 8: 51, 1994.
5. Pettersson, K., Piironen, T., Seppala, M., Liukkonen, L., Christensson, A., Matikainen, M.-T., Suonpaa, M., Lovgren, T. and Lilja, H.: Free and complexed prostate-specific antigen (PSA): in vitro stability, epitope map, and development of immunofluorometric assays for specific and sensitive detection of free PSA and PSA-alpha 1-antichymotrypsin complex. *Clin. Chem.*, 41: 1480, 1995.
6. Lilja, H., Christensson, A., Dahlen, U., Matikainen, M.-T., Nilsson, O. and Pettersson, K.: Prostate-specific antigen in serum occurs predominantly in complex with alpha 1-antichymotrypsin. *Clin. Chem.*, 37: 1618, 1991.
7. Christensson, A., Laurell, C.-B. and Lilja, H.: Enzymatic activity of prostate-specific antigen and its reaction with extracellular serine proteinase inhibitors. *Eur. J. Biochem.*, 194: 755, 1990.
8. Sottrup-Jensen, L.: alpha-Macroglobulins: structure, shape, and mechanisms of proteinase complex formation. *J. Biol. Chem.*, 264: 11539, 1989.
9. LaMarre, J., Wollenberg, G. K., Gonias, S. L. and Hayes M. A.: Biology of disease: cytokine binding and clearance properties of proteinase-activated alpha 2-macroglobulins. *Lab. Invest.*, 65: 3, 1991.
10. Kratzsch, J., Selisko, T. and Birkenmeir, G.: Identification of transformed alpha 2-macroglobulin as a growth hormone binding protein in human blood. *J. Clin. Endocrinol. Metab.*, 80: 585, 1995.
11. Moestrup, S. K., Gliemann, J. and Pallessen, G.: Distribution of the alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein in human tissues. *Cell Tissue Res.*, 269: 375, 1992.
12. Herz, J., Hamann, U., Rogne, S., Myklebost, O., Gausepohl, H. and Stanley, K. K.: Surface location and high affinity for calcium of a 500-kDa liver membrane protein closely related to the LDL-receptor suggest a physiological role as lipoprotein receptor. *EMBO J.*, 7: 4119, 1989.
13. Strickland, D. K., Ashcom, J. D., Williams, S., Burgess, W. H., Migliorini, M. and Argraves, W. S.: Sequence identity between the alpha 2-macroglobulin receptor and low density lipoprotein receptor-related protein suggests that this molecule is a multifunctional receptor. *J. Biol. Chem.*, 265: 17401, 1990.
14. Strickland, D. K., Kounnas, M. Z. and Argraves, W. S.: LDL receptor-related protein: a multiligand receptor for lipoprotein and proteinase catabolism. *FASEB J.*, 9: 890, 1995.
15. Van Leuven, F., Cassiman, J.-J. and Van den Berghe, H.: Functional modifications of alpha 2-macroglobulin by primary

- amines. J. Biol. Chem., 256: 9016, 1981.
- 16. Birkenmeier, G. and Stigbrand, T.: Production of conformation-specific monoclonal antibodies against alpha 2-macroglobulin and their use for quantitation of total and transformed alpha 2-macroglobulin in human blood. J. Immunol. Meth., 162: 59, 1993.
 - 17. Laemmli, U. K.: Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature, 227: 680, 1970.
 - 18. Hunter, W. M. and Greenwood, F. C.: Preparation of iodine-131 labeled human growth hormone of high specific radioactivity. Nature (London), 194: 495, 1962.
 - 19. Gettings, P., Beth, A. H. and Cunningham, L. W.: Proximity of thiol esters and bait region in human alpha 2 macroglobulin: paramagnetic mapping. Biochemistry, 27: 2905, 1988.
 - 20. Feinman, R. D., Yuan, A. I., Windwer, S. R and Wang, D.: Kinetics of the reaction of thrombin and alpha 2-macroglobulin. Biochem. J., 231: 417, 1985.
 - 21. Leinonen, J., Zhang, W.-M. and Stenman, U.-H.: Complex formation between PSA isoenzymes and protease inhibitors. J. Urol., 155: 1099, 1996.
 - 22. Wu, J. T., Wilson, L., Zhang, P., Meikle, A. W. and Stephenson, R.: Correlation of serum concentration of PSA-ACT complex with total PSA in random and serial specimens from patients with BPH and prostate cancer. J. Clin. Lab. Anal., 9: 15, 1995.
 - 23. Agha, A. H., Schechter, E., Roy, J. B. and Culkin, D. J.: Prostate specific antigen is metabolized in the liver. J. Urol., 155: 1332, 1996.
 - 24. Cohen, P., Graves, H. C. B., Peehl, D. M., Kamarei, M., Giudice, L. C. and Rosenfeld, R. G.: Prostate-specific antigen (PSA) is an insulin-like growth factor binding protein-3 protease found in seminal plasma. J. Clin. Endocrinol. Metab., 75: 1046, 1992.
 - 25. Glander, H.-J., Kratzsch, J., Weisbrich, Ch. and Birkenmeier, G.: Insulin-like growth factor-I (IGF-I) and alpha 2-macroglobulin (alpha 2-M) in seminal plasma correlate with semen quality. Hum. Reprod., 11: 2454, 1996.
 - 26. McGarvey, T., Hussain, M. M. and Stearn, M. E.: In situ hybridization of alpha 2-macroglobulin receptor and receptor-associated protein in human prostate carcinoma. Prostate, 28: 311, 1996.

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